EX-3

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4. The theory of equilibrium condensation, in which the nebular temperature gradient controls the composition of condensates, the inner boundary of hydrated silicate condensation at a heliocentric distance between the orbits of Earth and Mars and predicts a much greater initial water abundance on Earth than on Venus [J. S. Lewis, *Icarus* 16, 241 (1972)]. Proponents of a wet young Venus argue that sufficient mixing occurred among the terrestrial planetesimals to provide them with nearly identical volatile abundances. However, the theory of planetary accretion is incomplete, and scenarios range from those in which planetesimals from a wide range of heliocentric distances are incorporated into each planet to those in which eccentricities remain low and a large degree of segregation is maintained [R. Greenberg, in *Planetary and Satellite Atmospheres: Origin and Evolution*, S. K. Atreya, J. B. Pollack, M. S. Matthews, Eds. (Univ. of Arizona Press, Tucson, in press)]. The observed differences in bulk density among the terrestrial planets must be the result of incomplete mixing among their precursors. The extent of such mixing should be regarded as an open question, awaiting more complete condensation-accretion models.
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Blocking of HIV-1 Infectivity by a Soluble, Secreted Form of the CD4 Antigen

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The initial event in the infection of human T lymphocytes, macrophages, and other cells by human immunodeficiency virus (HIV-1) is the attachment of the HIV-1 envelope glycoprotein gp120 to its cellular receptor, CD4. As a step toward designing antagonists of this binding event, soluble, secreted forms of CD4 were produced by transmembrane and cytoplasmic domains. The soluble CD4 so produced binds gp120 with an affinity and specificity comparable to intact CD4 and is capable of neutralizing the infectivity of HIV-1. These studies reveal that the high-affinity CD4-gp120 interaction does not require other cell or viral components and may establish a novel basis for therapeutic intervention in the acquired immune deficiency syndrome (AIDS).

THE PRIMARY IMMUNOLOGIC ABnormality resulting from infection by the human immunodeficiency virus (HIV-1) is the progressive depletion and functional impairment of T cells expressing the CD4 antigen (*1*). CD4 is a nonpolymorphic surface glycoprotein with partial sequence identity to immunoglobulins (*2*). The CD4 and CD8 antigens define distinct subsets of mature peripheral T cells, whose ability to interact with antigen is restricted by the expression of class II and class I major histocompatibility antigens on the antigen-presenting cell, respectively (*3*). Most $CD4^+$ T cells have helper/inducer function, although some have cytotoxic/suppressor activity, usually associated with $CD8^+$ T cells (*4*). The ability of HIV-1 to selectively infect, replicate in, and destroy $CD4^+$ T cells in part explains the loss of $CD4^+$ helper/inducer function characteristic of AIDS (*5*). That the CD4 molecule itself is the cellular receptor for HIV was first suggested by the ability of antibodies to CD4 to block HIV-1 infection and syncytia induction (*6*), and confirmed by the detection of CD4 binding to gp120, the major envelope glycoprotein of HIV-1 (*7*), and the finding that nonpermissive human cells are rendered susceptible to HIV-1 infection after the stable expression of a CD4 complementary DNA (cDNA) (*8*). The interaction of CD4 with gp120 also plays a critical role in the formation of multinucleated giant cells by cell fusion, the major cytopathic effect induced by HIV-1 (*9*, *10*). Fusion of uninfected $CD4^+$ cells with HIV-1 infected cells is blocked by antibodies to CD4 (*9*); furthermore, cells producing HIV-1 *env* polypeptides in the absence of other viral gene products efficiently induce the fusion of uninfected $CD4^+$ cells (*11*). Given its essential role, the interaction of gp120 with CD4 is probably not significantly affected by genetic variation among HIV isolates (*12*), and thus provides an attractive approach to therapeutic intervention against HIV-1 (*13*). One successful strategy for the treatment of receptor-mediated abnormalities has been the design of antagonists that block binding of the natural ligand (*14*). To begin development of inhibitors of HIV-1 attachment and gp120-mediated cell fusion, we have produced soluble CD4 analogs with an affinity for gp120 comparable to that of intact CD4. The ability of soluble CD4 to efficiently neutralize HIV-1 infection of $CD4^+$ cells in vitro suggests the potential application of such molecules in the treatment of AIDS and related conditions.

The CD4 precursor consists of a signal peptide, a 370–amino acid extracellular region containing four immunoglobulin-like domains, a membrane-spanning domain, and a charged intracellular region of 40 residues (*2*) (Fig. 1). We previously expressed the intact CD4 antigen in Chinese hamster ovary (CHO) cells under the transcriptional control of the SV40 early promoter; the CD4 expressed by these cells is located on the cell surface and binds gp120 (*15*). To produce soluble versions of CD4, we replaced the transmembrane domain, which presumably anchors the molecule to the cell surface, and the cytoplasmic domain, with a short linker sequence containing an in-frame stop codon. The resulting truncated CD4-encoding cDNAs were inserted into expression vectors (*15*) and examined for the ability to direct the biosynthesis and secretion of CD4 antigens. Two constructs were studied: the first included the natural signal sequences of CD4 itself (CD4T), while in the second the CD4 leader was replaced by the signal peptide and first 27 amino acids of the glycoprotein D (gD) of herpes simplex virus type I (GDCD4T) (Fig. 1).

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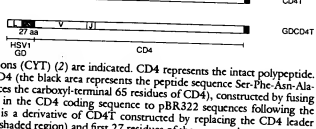
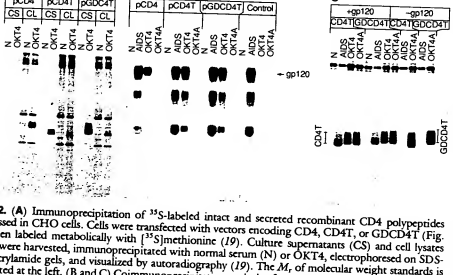
Cells transfected with plasmids encoding intact CD4 or either truncated polypeptide were metabolically labeled with [^{35}S]methionine; cell lysates and clarified cell culture medium were then analyzed by immunoprecipitation with monoclonal antibodies reactive with CD4. Vectors encoding intact CD4 directed the synthesis of a cell-associated 55-kD polypeptide, with no evidence for CD4-related antigen detectable in the culture fluid (Fig. 2A). In marked contrast, the CD4 polypeptides produced by cells transfected with vectors encoding CD4T and GDCD4T were present largely in the culture media, although in each case a faster migrating putative intracellular precursor protein was detected in cell lysates (Fig. 2A). Whereas a single diffuse band was observed for intact CD4, suggesting heterogeneity due to glycosylation, CD4T and GDCD4T were represented by three distinct species of M_r 51,000–55,000 and 53,000–57,000, respectively (Fig. 2A). Notably, each GDCD4T band was 2 kD larger than its CD4T counterpart, confirming the presence of the gD-derived residues.

To determine whether soluble CD4 is capable of binding gp120, we used a coimmunoprecipitation assay to demonstrate the formation of CD4-gp120 complexes (*7*). ^{35}S-Labeled recombinant gp120, prepared by metabolic labeling of CHO cells secreting a soluble form of gp120 (*16*), was incubated either with detergent-solubilized intact CD4, supernatants from cells secreting CD4T or GDCD4T, or supernatants from mock-transfected cells. After the binding reaction, immunoprecipitations were carried out with gp120-reactive serum from an AIDS patient or with the monoclonal antibody OKT4, which recognizes CD4-gp120 complexes (*7*). As a control for the specificity of complex detection, precipitations were also carried out with the monoclonal antibody OKT4A, which blocks the binding of HIV-1 to CD4, and thus lacks the ability to precipitate CD4-gp120 complexes (*7*). After incubation of rgp120 with solubilized intact CD4 or either soluble CD4 analog, OKT4 coprecipitated a level of ^{35}S-rgp120 similar to that detected by direct precipitation with AIDS serum; in contrast, no rgp120 precipitate was observed after incubation with supernatant from mock-transfected cells (Fig. 2B). Neither OKT4A nor normal serum coprecipitated rgp120 after incubation with intact or secreted CD4, confirming that precipitation of rgp120 by OKT4 reflected the formation of CD4-gp120 complexes (Fig. 2B). To provide additional confirmation for the interaction of rgp120 and soluble CD4, we conducted coprecipitation experiments with ^{35}S-labeled soluble CD4 and unlabeled rgp120. Serum from the AIDS patient coprecipitated soluble CD4 polypeptides only if rgp120 was included in the binding reaction (Fig. 2C).

To determine the affinity constant for this interaction, we carried out saturation binding analysis with soluble CD4 and detergent-solubilized intact CD4 using radioiodinated rgp120. So that the data for soluble and intact molecules would be comparable, the nonionic detergent NP40 was included in binding reactions at the concentration used to release intact CD4 from cells. All three forms of the CD4 molecule were bound saturably by rgp120, and yielded a simple mass action binding curve (Fig. 3). Supernatants from mock-transfected cells gave a level of specifically bound rgp120 less than 1% that observed with supernatants containing soluble CD4. Scatchard analysis revealed a single class of gp120 binding sites on each molecule, with apparent dissociation constants (K_d) of $1.3 \times 10^{-9}M$, $0.83 \times 10^{-9}M$, and $0.72 \times 10^{-9}M$ for CD4, CD4T, and GDCD4T, respectively. The values obtained for CD4-gp120 binding in solution are comparable to the affinity



ig. 1. Structure of intact and ancated derivatives of D4 polypeptide. The numbers the top indicate the position thin the CD4 amino acid sequence, starting at the amino minus of mature CD4. The der sequence (*L*), immunobulin variable (*V*) and join- (*J*)-like regions, transmembane (TM), and cytoplasmic regions (CYT) (*2*) are indicated. CD4 represents the intact polypeptide. D4T is a truncated analog of CD4 (the black area represents the peptide sequence Ser-Phe-Asn-Ala-l-Val-Tyr-His-Ser, which replaces the carboxyl-terminal 65 residues of CD4), constructed by fusing ique Hind III site. GDCD4T is a derivative of CD4T constructed by replacing the CD4 leader uence with the signal peptide (shaded region) and first 27 residues of the mature glycoprotein D of V-1 (cross-hatched region).



2. (**A**) Immunoprecipitation of ^{35}S-labeled intact and secreted recombinant CD4 polypeptides essed in CHO cells. Cells were transfected with vectors encoding CD4, CD4T, or GDCD4T (Fig. ien labeled metabolically with [^{35}S]methionine (*19*). Culture supernatants (*CS*) and cell lysates were harvested, immunoprecipitated with normal serum (*N*) or OKT4, electrophoresed on SDS-crylamide gels, and visualized by autoradiography (*19*). The M_r of molecular weight standards is ated at the left. (**B** and **C**) Coimmunoprecipitation analysis of rgp120 binding to intact or secreted polypeptides. (**B**) Coimmunoprecipitation of ^{35}S-labeled rgp120 with unlabeled intact and and incubated with detergent-solubilized cells expressing intact CD4 (pCD4) or culture natants from cells secreting soluble CD4 (pCD4T or pGDCD4T), or mock-transfected cells rol) (*21*). Binding reactions were then immunoprecipitated with normal serum (*N*), serum from DS patient with high antibody titers to gp120 (*AIDS*), or OKT4 or OKT4A, and the precipitated ns resolved on SDS-polyacrylamide gels. The position of gp120 is indicated by the arrow. (**C**) cipitation of ^{35}S-labeled soluble CD4 with unlabeled rgp120. Transfected cells secreting soluble (CD4T or GDCD4T) were labeled with [^{35}S]methionine (*19*). Supernatants containing ^{35}S-.20) or supernatants from untransfected cells (−gp120). Binding reactions were then analyzed by ipitation as described above. The positions of the CD4T and GDCD4T polypeptides are ed by brackets. The aberrant mobility of CD4T and GDCD4T observed in the AIDS lanes is a bility artifact due to the higher level of immunoglobulin G heavy chain present in the AIDS sera n the other sera, which comigrates with CD4.

we have measured for rgp120 binding to CD4 on whole cells ($K_d = 4.0 \times 10^{-9} M$) (*15*).

Given the high affinity of soluble CD4 for gp120, we examined its ability to block HIV-1 infection in vitro. One hundred $TCID_{50}$ units (50% tissue culture infective dose) of HIV-1 isolate $HTLV\text{-}III_B$ were incubated with supernatants containing CD4T or GDCD4T, or supernatants from mock-transfected cells, and then added to cultures of the $CD4^+$ H9 human T cell line (*17*); incubation in the presence of the appropriate supernatant was continued for 3 days. After 7 days, cells were analyzed by indirect immunofluorescence with the use of a serum with high antibody titers to HIV-1 proteins, and culture supernatants were assayed for reverse transcriptase activity. Both forms of soluble CD4 virtually abolished the growth of HIV-1 when incubated with virus-infected cells without prior dilution (Table 1). At a dilution of 1:4 the soluble CD4 preparations were only partly effective in inhibiting virus growth; however, the levels of fluorescence-positive cells and reverse transcriptase were still significantly lower than in cultures receiving mock-transfected cell supernatant. Since there was no significant difference in virus growth between diluted and undiluted control supernatants, and since none of the supernatants affected the growth of uninfected H9 cells, we conclude that soluble CD4 was responsible for the neutralization of HIV-1.

These results show that the gp120 binding domain of CD4 lies in its extracellular region and that high-affinity binding occurs in the absence of other components on $CD4^+$ T cells and HIV-1. The ability of soluble CD4 to prevent infection by free HIV-1 probably results from the saturation of virion-associated gp120 with soluble receptor, thus interfering with the adsorption of virus to the cell. While it is premature to assess the clinical significance of these findings, it is possible to conceive of therapeutic modalities in which soluble CD4 could alter the pathogenesis of HIV infection. In addition to restricting the dissemination of infection by released virus, soluble CD4 might exert a benefit by sequestering free gp120 shed by HIV-1–infected cells. For example, Lyerly *et al.* (*18*) have shown that uninfected $CD4^+$ lymphocytes that have bound gp120 become targets for antibody-dependent cellular cytoxicity. Soluble CD4 might also be capable of inhibiting the cell-fusing activities of HIV-1 which play an important role in the formation of multinucleated giant cells and the spread of infection by cell-cell fusion and appear to require a CD4-gp120 interaction (*6, 9, 11*). Sera from HIV-1–infected individuals, while capable of neutralizing free virus infectivity, have only a limited capacity to inhibit HIV-1–induced cell fusion (*11*). This limitation may be the consequence of the moderate degree of sequence heterogeneity found at a putative CD4 contact site within gp120 (*15*). In this respect, functional constraints on gp120 structure may bestow a critical advantage on soluble CD4 over neutralizing antibodies in its ability to block the CD4-gp120 interation.

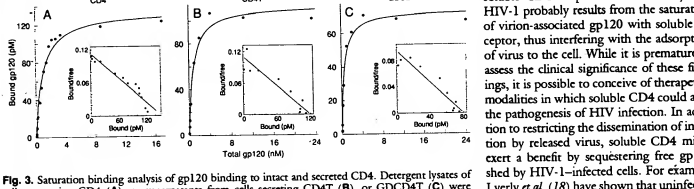


Fig. 3. Saturation binding analysis of gp120 binding to intact and secreted CD4. Detergent lysates of cells expressing CD4 (**A**), or supernatants from cells secreting CD4T (**B**), or GDCD4T (**C**) were incubated with increasing concentrations of pure rgp120 (*16*) radioiodinated with lactoperoxidase (*22*). CD4-gp120 complexes were quantitated by immunoprecipitation with OKT4 (Ortho) (*21*). Specifically bound ^{125}I-gp120 was determined from the difference in binding in the presence and absence of 50 μg of unlabeled rgp120 (≥74-fold excess), and is plotted against the total ^{125}I-gp120 concentration (*22*). The curves represent the best fit determined by unweighted least-squares nonlinear regression analysis. Scatchard analysis of the data is shown in the insets; the line drawn represents the best fit as determined by unweighted least squares linear regression analysis. The dissociation constants (K_d) thus obtained for rgp120 binding to CD4T, GDCD4T and intact CD4 were $(0.84 \pm 0.10) \times 10^{-9} M$, $(0.72 \pm 0.13) \times 10^{-9} M$, and $(1.3 \pm 0.11) \times 10^{-9} M$, respectively (data presented are the mean ± SEM of two experiments).

Table 1. Neutralization of HIV-1 infectivity by soluble CD4 analogs. A modification of the neutralization procedure of Robert-Guroff *et al.* (*28*) was followed. Equal volumes of inhibitor supernatant and virus (60 μl) were incubated at 4°C for 1 hour, then the same volume of H9 cells (*19*) at 5×10^6 per milliliter was added and incubation continued for 1 hour at 37°C. After adsorption, 2.5×10^5 cells in 150 μl were transferred to 2 ml of incubation media. After 4 days at 37°C, the cultures were split 1:2 with fresh medium and incubated for an additional 3 days. An equal portion of each culture was harvested, and reverse transcriptase activity and immunofluorescence reactivity with HIV-1–positive serum were determined as described (*18*). Inhibitor supernatants consisted of transfected cell cultures or mock–transfected cultures, in RPMI 1640 medium containing 20% fetal calf serum. Inhibitor supernatant replaced part or all of the incubation medium during the first 3 days of culture as indicated. Challenge dose of virus was 100 $TCID_{50}$ of HIV-1 strain $HTLV\text{-}III_B$ grown in H9 cells (*19*) assayed in the same system. The incubation medium was RPMI 1640 containing 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 μg/ml of polybrene, and 20% fetal calf serum (M.A. Bioproducts).

Inhibitor supernatant	Dilution	Indirect immunofluorescence (% positive cells)	Reverse transcriptase (cpm/ml × 10^5)
Mock-transfected	Undiluted	65.3	21.8
	1:4	65.5	23.9
Mock-transfected	Undiluted	61.2	18.5
	1:4	61.1	28.1
CD4T	Undiluted	0.4	0.11
	1:4	18.0	5.94
CD4T	Undiluted	0.8	0.15
	1:4	16.1	3.72
GDCD4T	Undiluted	0.4	0.14
	1:4	26.8	9.92
GDCD4T	Undiluted	1.4	0.23
	1:4	36.1	11.3

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9. Cells were transfected with plasmids encoding CD4, CD4T, or GDCD4T by a modification of the calcium phosphate procedure (*20*). Confluent 60-mm plates were labeled 48 hours later with 1.2 ml of methionine-free DME medium containing 1 mCi of [^{35}S]methionine (1200 Ci/mmol) for 6 hours. Culture supernatants were collected, clarified by low speed centrifugation, and diluted with an equal volume of 2× RIPA buffer, cells were washed with phosphate-buffered saline and then dissolved in 1 ml of 1× RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.12*M* NaCl, 0.05*M* tris-HCl, *p*H 7.5). Samples were preadsorbed with 5 μl of normal rabbit serum for 1 hour at 4°C and cleared with 40 μl of Pansorbin (10% w/v, Calbiochem) for 30 minutes at 4°C. Samples were then incubated overnight at 4°C with 2 μl of normal serum or 5 μl (0.25 μg) of OKT4 (Ortho Diagnostics), and immune complexes were then collected with 10 μl of Pansorbin. Precipitates were washed twice in 1× RIPA and once in water, then eluted by heating at 100°C for 2 minutes in sample buffer (0.12*M* tris-HCl, *p*H 6.8, 0.7*M* β-mercaptoethanol, 20% glycerol, 4% SDS, and 0.1% bromophenol blue). Immunoprecipitated proteins were resolved on SDS polyacrylamide gels and visualized by fluorography (*20*).
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Coimmunoprecipitation of CD4-gp120 complexes was performed by a modification of the procedure of McDougal *et al.* (*7*). Binding reactions consisted of ^{35}S-labeled rgp120 incubated at 4°C for 1 hour with either lysates of cells expressing intact CD4 or supernatants from cells containing CD4T or GDCD4T or from mock-transfected cells. Alternatively, supernatants containing ^{35}S-labeled CD4T or GDCD4T were incubated with supernatants from cells containing unlabeled rgp120 (*16*) or untransfected cells. Binding reactions had a final composition of 0.5× McDLB (1× McDLB is 0.5% NP40, 0.2% sodium deoxycholate, 0.12*M* NaCl, 0.02*M* tris-HCl, *p*H 8.0). Reactions were immunoprecipitated and analyzed as described above (*24*) except that washes were carried out with 1× McDLB. Purified soluble rgp120 (*16*) was radioiodinated with lactoperoxidase to a specific activity of 2.9 nCi/ng. Binding reactions consisted of ^{125}I-gp120 (3 ng to 670 ng) incubated for 1 hour at 4°C with cell lysates containing intact CD4 or cell supernatants containing unlabeled CD4T or GDCD4T (*19*). Reactions (0.2 ml) had a final composition of 0.5× McDLB, and were performed in duplicate, both in the presence or absence of 50 μg of unlabeled purified rgp120 (*16*). After incubation, bound gp120 was quantitated by immunoprecipitation (*19*), and counted in a gamma counter. Data were analyzed by the Scatplot program written by R. Vandlen, Genentech, Inc.
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We thank E. Peralta, J. Winslow, and P. Moore for discussions; C. Lucas and S. Frei for providing ^{125}I-gp120; M. Vasser, P. Jurhani, and P. Ng for oligodeoxynucleotide synthesis; and C. Morris and W. Anstine for preparation of the figures.

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Three-Dimensional Structure of Interleukin-2

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Interleukin-2 is an effector protein that participates in modulating the immune response; it has become a focal point for the study of lymphokine structure and function. The three-dimensional structure of the interleukin molecule has been solved to 3.0 angstrom resolution. Interleukin-2 has a novel alpha-helical tertiary structure that suggests one portion of the molecule forms a structural scaffold, which underlies the receptor binding facets of the molecule.

INTERLEUKIN-2 (IL-2) IS A LYMPHOKINE protein produced by antigen- or mitogen-stimulated T lymphocytes whose first documented activity is to stimulate proliferation of IL-2–dependent T cells (*1*). It modulates immunological effects on cytotoxic T cells (*2*, *3*), natural killer cells (*4*, *5*), activated B cells (*6*, *7*) and lymphokine-activated cells (*8*, *9*). IL-2 asserts its effect by binding a specific high affinity receptor on the surface of target cells; consequently, the IL-2 molecule has become a focal point for studying receptor-effector interactions that modulate cell proliferation in the immune response.

The high affinity ($K_D \sim 10^{-11}M$) receptor responsible for mediating the effect of IL-2 on target cells consists of two distinct membrane-bound proteins of size 55 kD (p55 or Tac) and 75 kD (p75); each of these two proteins can act by itself as an apparent low affinity ($K_D \sim 10^{-8}M$) receptor for IL-2, and both are required for IL-2 activity (*10*). This suggests that IL-2 must simultaneously bind both p55 and p75 to form a trimeric complex for activity, and by inference, that IL-2 must have two separate receptor binding sites.

Knowledge of the three-dimensional structure of IL-2 should provide a foundation for systematic delineation of its receptor binding sites, as well as an evaluation of the accuracy of recent predictions of its structure (*11*, *12*). We reported earlier the low (5.5 Å) resolution structure of IL-2 (*13*); we have extended these crystallographic results to 3.0 Å resolution, and now describe the overall tertiary structure of IL-2.

The purification, crystallization, and initial structure determination to 5.5 Å resolution of human recombinant IL-2, as well as structural work reported by others, have been described (*13*). Briefly, we have found that IL-2 and an analog in which Cys^{125} has been replaced with alanine ([Ala^{125}]IL-2) crystallize isomorphously in the triclinic space group *P*1, with unit cell parameters $a = 55.8$ Å, $b = 40.1$ Å, $c = 33.7$ Å, $\alpha = 90.0°$, $\beta = 109.3°$, and $\gamma = 93.2°$. The unit cell contains two molecules related by near-2_1 symmetry. With rare exception, an unusual twinning of the crystals leads to overlap of reflections from the two contributing twins for reciprocal lattice indices $k = 0, \pm 6, \pm 7, \pm 12, \pm 13, \ldots$.

Data were collected by diffractometer, processed, and scaled (*13*); multiple heavy atom isomorphous replacement (MIR) phasing statistics are summarized in Table 1. Two crystals that were essentially single (showed no significant twinning) allowed us to collect data on reflections that overlap in twinned crystals for the native and mercury phenyl glyoxal data sets in [Ala^{125}]IL-2 crystals to 3.5 Å resolution. Reflections that overlapped because of the crystal twinning for the other heavy atom derivatives were discarded from the computations.

Solvent flattening was used to improve the quality of the 3.0 Å phases. In our implementation of the method, envelopes outlining each molecule in the unit cell were constructed and digitized; the envelope encompassed 27.3 percent and 27.0 percent of the cell volume for the first and second molecule, respectively, leaving 45.7 percent as solvent. Iteratively, maps were computed on a 1 Å grid, the solvent density was set equal to its average value, and the protein density was left unmodified; the solvent-flattened map was Fourier-transformed to produce calculated structure factors (F_{calc}), the $|F_{calc}|$'s were scaled in shells of resolution to the measured native structure factor magnitudes ($|F_{obs}|$), and Sim-weighted calculated phases were combined with MIR phases by direct multiplication of their phase probability distributions to yield combined phases. The combined phases were then used to calculate a new map.

After several cycles, the overall agreement between observed and calculated structure factors,

$$R = \Sigma||F_{calc}| - |F_{obs}||/\Sigma|F_{obs}|$$

summed over all contributing reflections,

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